

The Activation and Inhibition of Human Nicotinic Acetylcholine Receptor by RJR-2403 Indicate a Selectivity for the $\alpha 4\beta 2$ Receptor Subtype

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Abstract: Human nicotinic acetylcholine (ACh) receptor subtypes expressed in *Xenopus* oocytes were characterized in terms of their activation by the experimental agonist RJR-2403. Responses to RJR-2403 were compared with those evoked by ACh and nicotine. These agonists were also characterized in terms of whether application of the drugs had the effect of producing a residual inhibition that was manifest as a decrease in subsequent control responses to ACh measured 5 min after the washout of the drug. For the activation of $\alpha 4\beta 2$ receptors, RJR-2403 had an efficacy equivalent to that of ACh and was more potent than ACh. RJR-2403 was less efficacious than ACh for other human receptor subtypes, suggesting that it is a partial agonist for all these receptors. Nicotine activated peak currents in human $\alpha 4\beta 2$ and $\alpha 3\beta 2$ receptors that were 85 and 50% of the respective ACh maximum responses. Nicotine was an efficacious activator of human $\alpha 7$ receptors, with a potency similar to ACh, whereas RJR-2403 had very low potency and efficacy for these receptors. At concentrations of <1 mM, RJR-2403 did not produce any residual inhibition of subsequent ACh responses for any receptor subtype. In contrast, nicotine produced profound residual inhibition of human $\alpha 4\beta 2$, $\alpha 3\beta 2$, and $\alpha 7$ receptors with IC_{50} values of 150, 200, and 150 μM , respectively. Co-expression of the human $\alpha 5$ subunit with $\alpha 3$ and $\beta 2$ subunits had the effect of producing protracted responses to ACh and increasing residual inhibition by ACh and nicotine but not RJR-2403. In conclusion, our results, presented in the context of the complex pharmacology of nicotine for both activating and inhibiting neuronal nicotinic receptor subtypes, suggest that RJR-2403 will be a potent and relatively selective activator of human $\alpha 4\beta 2$ receptors. **Key Words:** Desensitization—Voltage clamp—Alzheimer's disease—Noncompetitive inhibition. *J. Neurochem.* 75, 204–216 (2000).

Brain nicotinic receptor systems have long been associated with addiction. Recently, it has been shown that nicotinic receptor systems may be involved with Tourette's syndrome (Silver et al., 1996; Sanberg et al.,

1998) and schizophrenia (Adler et al., 1992, 1993; Leonard et al., 1993; Freedman et al., 1994) and that nicotinic drugs may also have applications as analgetics and for the treatment of Alzheimer's disease (Williams et al., 1994; Armeric et al., 1995). With these newly defined therapeutic endpoints, we are presented with the challenge of understanding how best to target nicotinic drugs to the receptor systems of the brain.

The pharmacology of neuronal nicotinic receptors, however, encompasses very complex issues on almost every level amenable to study. With a gene family that includes at last eight different α subunits (designated $\alpha 2$ – $\alpha 9$) that in some cases may function as homooligomers ($\alpha 7$ – $\alpha 9$) or alternatively combine with different neuronal β subunits ($\beta 2$ – $\beta 4$), there is a great potential for structural diversity just on the level of the basic pentamer receptor subunit combinations (Papke, 1993). Multiple receptor subtypes are commonly found on single neurons, and single tissues have multiple neuronal cell types that differ in the function of their nicotinic receptors (Mulle et al., 1991). Likewise, nicotinic receptor expression differs widely from one brain region to another, with the connections between diverse receptors in multiple brain regions and complex behaviors associated with nicotinic receptor functions still unclear. In addition to consideration of the complex nature of the neuronal substrates of the brain's nicotinic systems, we must also consider that nicotine, the prototype agonist, as well as many new experimental agents have a compli-

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Abbreviations used: ACh, acetylcholine; nAChR, nicotinic ACh receptor; RJR-2403, (E)-N-methyl-4-(3-pyridinyl)-3-butene-1-amine.

cated pharmacological profile that can include both the activation and the inhibition of diverse receptor subtypes.

One approach for sorting out significant elements in this complex system is to study cloned receptor subunits in defined combinations. In this article, we report the effects of a promising new experimental agonist RJR-2403 [(*E*)-*N*-methyl-4-(3-pyridinyl)-3-butene-1-amine or metanicotine] (Bencherif et al., 1996; Lippiello et al., 1996) on combinations of human neuronal nicotinic receptor subunits expressed in *Xenopus* oocytes. We compare the effects of RJR-2403 with those of nicotine as well as the endogenous agonist acetylcholine (ACh). The co-expression of $\alpha 4$ and $\beta 2$ subunits represents one receptor subunit combination of particular interest, as the primary high-affinity nicotinic receptor of the brain is composed of these subunits (Whiting and Lindstrom, 1986; Flores et al., 1992). Receptors containing the $\alpha 3$ subunit are also likely to be found in the brain but predominate in the peripheral nervous system (Halvorsen and Berg, 1990). The properties of both brain and ganglionic nicotinic ACh receptor (nAChR) can be modified by the co-assembly with the nonessential $\alpha 5$ subunit (Conroy et al., 1992; Wang et al., 1996; Gerzanich et al., 1998). Therefore, we have focused our studies on the pairwise combinations of $\alpha 3$ and $\alpha 4$ subunits with $\beta 2$ and $\beta 4$ subunits but also examined the triple subunit combination $\alpha 3\beta 2\alpha 5$, as in this combination the functional effects of $\alpha 5$ expression can easily be confirmed (Wang et al., 1996). We further extended our analysis to include another important type of brain nicotinic receptor subtype, those that bind α -bungarotoxin with high affinity. These receptors correspond to the $\alpha 7$ subunit gene products, which form homomeric receptors with high calcium permeability and fast desensitization to high concentrations of agonist.

MATERIALS AND METHODS

Chemicals

RJR-2403 was synthesized as previously described (Bencherif et al., 1996; Lippiello et al., 1996). (–)-Nicotine and all other chemicals for electrophysiology were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Stock 100 mM solutions of RJR-2403 and nicotine were made up in Ringer's solution and kept frozen. Fresh ACh stock solutions were made daily in Ringer's solution.

Preparation of RNA and expression in *Xenopus* oocytes

The human cDNAs were obtained from Dr. Jon Lindstrom (University of Pennsylvania) and have been previously characterized (Peng et al., 1994; Wang et al., 1996; Olale et al., 1997). RNA transcripts were prepared in vitro using the appropriate mMessage mMachine kit from Ambion (Austin, TX, U.S.A.) after linearization and purification of cloned cDNAs. For each batch of oocytes, two to three ovarian lobes were surgically removed from a *Xenopus laevis* female (Nasco, Ft. Atkinson, WI, U.S.A.). The ovarian tissue was then cut open to expose the oocytes and treated with collagenase (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 10 mM HEPES,

pH 7.6, 0.33 mM $MgSO_4$, 0.1 mg/ml gentamicin sulfate). Subsequently, stage 5 oocytes were isolated and injected with 50 nl each of a mixture of the appropriate subunit cRNAs (5–10 ng total). Recordings were made 1–7 days after injection.

Electrophysiology

Oocyte recordings were made with a Warner Instruments (Hamden, CT, U.S.A.) OC-725C oocyte amplifier and RC-8 recording chamber interfaced to a Macintosh personal computer. Data were acquired using Labview software (National Instruments) and filtered at a rate of 6 Hz for all subtypes other than $\alpha 7$. Data obtained from $\alpha 7$ -expressing oocytes were filtered at 20 Hz. Oocytes were placed in a Warner recording chamber with a total volume of 0.6 ml and perfused at room temperature by frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, pH 7.3, 1.8 mM $CaCl_2$) containing 1 μM atropine to block potential muscarinic responses. A Mariotte flask filled with Ringer's solution was used to maintain a constant hydrostatic pressure for drug deliveries and washes. It is known that at physiological concentrations such as is used in the present experiments, calcium will influence the responses of nAChR expressed in *Xenopus* oocytes. Calcium may act directly through an effect on channel gating and indirectly via the secondary activation of calcium-dependent chloride currents. However, we have previously shown that the calcium effects tend to produce an essentially linear amplification of responses and do not distort concentration–response relationships (Papke et al., 1997).

Drugs were diluted in perfusion solution and loaded into a 2-ml loop at the terminus of the perfusion line. A bypass of the drug-loading loop allowed bath solution to flow continuously while the drug loop was loaded, and then drug application was synchronized with data acquisition by using a two-way electronic valve. Current electrodes were filled with a solution containing 250 mM CsCl, 250 mM CsF, and 100 mM EGTA and had resistances of 0.5–2 M Ω . Voltage electrodes were filled with 3 M KCl and had resistances of 1–3 M Ω . Oocytes with resting membrane potentials more positive than –30 mV were not used.

Current responses to drug application were studied under two-electrode voltage clamp at a holding potential of –50 mV. Holding currents immediately prior to agonist application were subtracted from measurements of response to agonist. All drug applications were separated by a wash period of 5 min unless otherwise noted. At the start of recording, all oocytes received an initial control application of ACh. Subsequent drug applications were normalized to the control ACh application to control for the level of channel expression in each oocyte. Means and SEM were calculated from the normalized responses of at least four oocytes for each experimental concentration. To measure residual inhibitory effects, the experimental applications of ACh, RJR-2403, and nicotine were followed by additional ACh control applications. These second controls were then compared with the preapplication control ACh responses. Cells were subjected to multiple drug applications only if follow-up control ACh responses were not decreased by >25% compared with the previous ACh control responses.

For most experiments, the rate of bath solution exchange and all drug applications was 6 ml/min. These conditions closely replicated those used for numerous previously published studies (e.g., de Fiebre et al., 1995; Papke et al., 1997; Meyer et al., 1998a,b), which provides a basis for direct comparisons to be made among these studies. However, due to the strong inhibitory (or desensitizing) effects of nicotine on $\alpha 4\beta 2$ receptors, we noted that this perfusion rate impacted our ability to esti-

mate the efficacy of nicotine for $\alpha 4\beta 2$ receptors based on peak currents (data not shown). Therefore, the nicotine concentration–response experiments on $\alpha 4\beta 2$ receptors (see Fig. 1) were conducted under conditions of more rapid perfusion (12 ml/min). Flow rate did not alter estimates of peak currents in other experiments. For example, under conditions of rapid perfusion, 30 μM ACh responses of $\alpha 4\beta 2$ receptors were $90 \pm 8\%$ of the amplitude of the 30 μM responses obtained with the normal solution perfusion. We also noted that perfusion rate had no apparent effect on net charge measurements, even for nicotine-evoked currents of $\alpha 4\beta 2$ receptors. Under the two perfusion conditions, measurements of net charge evoked by the application of 30 μM nicotine were 0.75 ± 0.07 and $0.82 \pm 0.06\%$ of the ACh-evoked net charge for the normal and fast perfusion rates, respectively. However, under conditions of rapid perfusion, recordings are relatively unstable and the values generated for peak responses are more variable than under our normal conditions. Therefore, for all experiments other than the nicotine concentration–response curves shown in Fig. 1, bath perfusion was kept at the normal rate of 6 ml/min.

Experimental protocols and data analysis

Measurements of net charge were made by integration of the current responses for 200 s after the initial deflection from baseline. Specifically, raw data values for experimental responses were imported into a template Excel spreadsheet along with the raw data for the corresponding ACh controls obtained 5 min prior to the experimental response. Each record included a short (0.5-s) interval of baseline that was used for offset correction. Peaks and areas were then calculated for both the experimental and the control responses, and the experimental values were expressed relative to their respective controls.

For the prolonged application experiments, drugs were applied by filling the chamber for 1 min with control or agonist solution and then maintaining the incubation in a static bath for 20 min. Following the 20-min incubation, the chamber was washed for 10 min before measuring an ACh control response. Data for ACh control peak responses as well as baseline drift were normalized to the initial ACh control obtained from the same cell.

In some experiments, the human $\alpha 5$ subunit was co-expressed along with the $\alpha 3$ and $\beta 2$ subunits. The $\alpha 5$ subunit is not required for function but has been reported to co-assemble with other subunits (Conroy et al., 1992). Because expression of the $\alpha 5$ subunit with the $\alpha 3$ and $\beta 2$ subunits causes an apparent increase in the potency of ACh for activation (Wang et al., 1996), it was possible to confirm expression of $\alpha 5$ for each injection set by measuring the ratio of response between 10 and 100 μM ACh. Injection sets in which the mean responses of four cells to 100 μM ACh were less than twice the response to 10 μM ACh were assumed to have good levels of $\alpha 5$ expression. In cells expressing $\alpha 3$ and $\beta 2$ alone, the ratio between 100 and 10 μM ACh responses was 4.1 ± 0.7 (cf. Figs. 2 and 5).

For concentration–response relations, data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software, Reading, PA, U.S.A.), and curves were generated using the Hill equation (Luetje and Patrick, 1991):

$$\text{response} = \frac{I_{\max}[\text{agonist}]^n}{[\text{agonist}]^n + (EC_{50})^n}$$

where I_{\max} denotes the maximal response for a particular agonist/subunit combination and n represents the Hill coefficient. I_{\max} , n , and the EC_{50} were all unconstrained for the fitting

procedures. Hill slopes of -1 were applied for the calculation of IC_{50} values.

RESULTS

RJR-2403: efficacious activator of human $\alpha 4\beta 2$ receptors

Oocytes expressing human $\alpha 4\beta 2$ subunits were tested for their responses to ACh, RJR-2403, and nicotine. The concentration–response relationships for these agonists, characterized in terms of the relative magnitude of the experimental responses compared with ACh controls from the same cells, are shown in Fig. 1A. Note that the nicotine responses and their respective controls were obtained under conditions of rapid perfusion (see Materials and Methods). Based on these measurements, RJR-2403 appears somewhat more potent than ACh and equally efficacious. Nicotine showed an efficacy that was $\sim 85\%$ that of ACh (see Tables 1 and 2).

Five minutes after the experimental applications, oocytes were rechallenged with another control ACh application. We found that the application of nicotine at concentrations of $\geq 30 \mu M$ caused a residual inhibition of the subsequent ACh response (Fig. 1B), whereas control responses were relatively unaffected by ACh and RJR-2403 applications. This apparent inhibition of $\alpha 4\beta 2$ receptors by nicotine suggests the existence of some form of channel block or persistent desensitization. However, as previously reported for rat $\alpha 4\beta 2$ receptors (de Fiebre et al., 1995), nicotine also produces a relatively prolonged activation of the human $\alpha 4\beta 2$ receptors such that even after 5 min of relatively rapid perfusion, the response evoked by 300 μM nicotine did not quite return to baseline (Fig. 1C, compare with ACh- and RJR-2403-evoked responses). Even with our normal perfusion conditions, our calculations of solution exchange (Papke and Thinschmidt, 1998) would predict about a millionfold dilution of the applied agonist after the 5-min wash. Such prolonged activation in the relative absence of agonist would seem to be inconsistent with desensitization per se and more consistent with prolonged effects of nicotine after it has bound to the receptor.

Dependence of apparent efficacy of RJR-2403 on both α and β subunits

As shown above, RJR-2403 is an effective activator of the $\alpha 4\beta 2$ neuronal nAChR subtype, with activity comparable with that of ACh. However, to establish a valuable context for the potential therapeutic applications of this compound, we sought to determine whether or not RJR-2403 displayed any significant selectivity for the activation of this receptor subtype. As shown in Fig. 2, compared with $\alpha 4\beta 2$ receptors, RJR-2403 shows an apparent reduction in efficacy and, to a lesser degree, potency when either the α or the β subunits are replaced with the alternative $\alpha 3$ or $\beta 4$ subunits (see Table 1). Moreover, of all the β subunit-containing receptor subtypes tested, RJR-2403 showed the lowest relative effi-

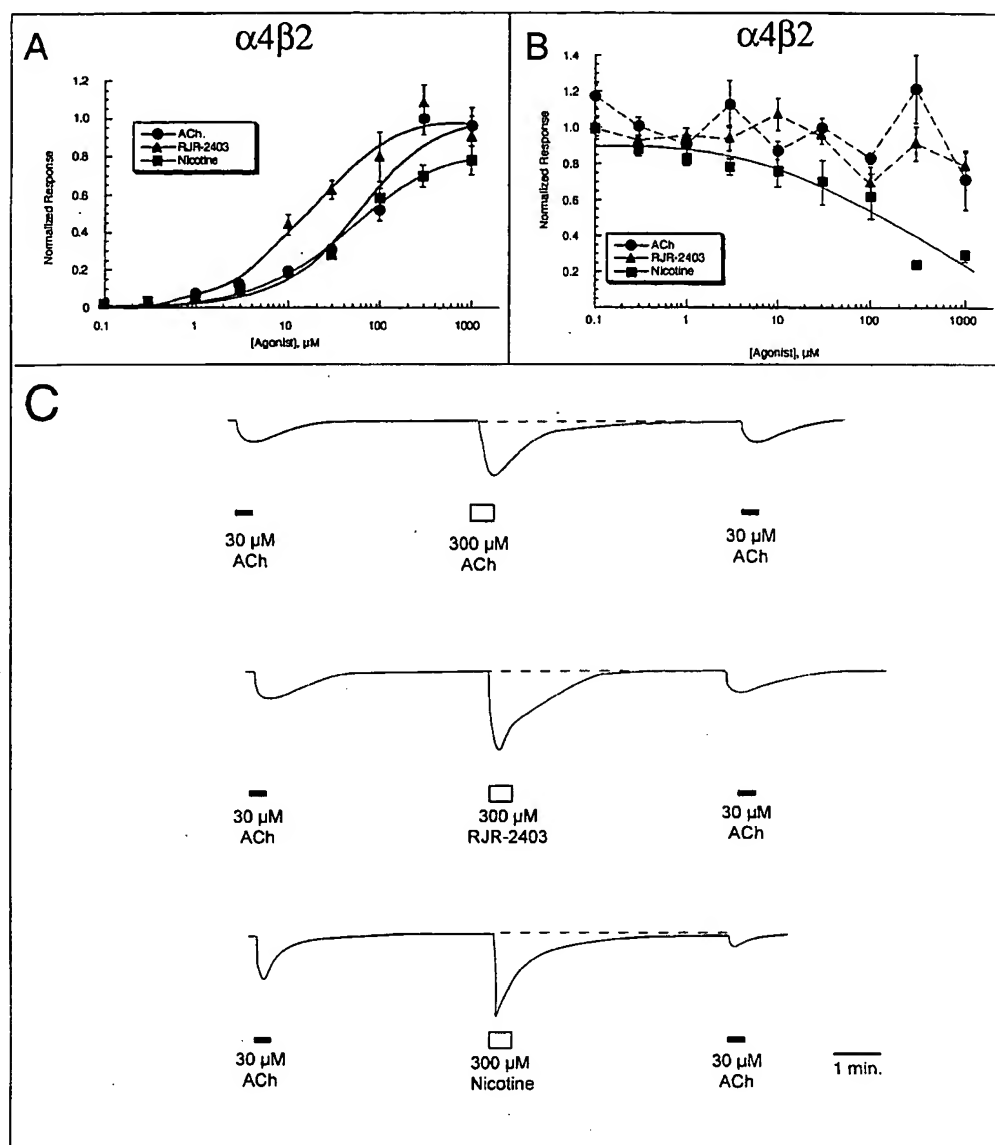


FIG. 1. **A:** Concentration–response curves for ACh, RJR-2403, and nicotine on human $\alpha 4\beta 2$ receptors expressed in *Xenopus* oocytes. Data from each oocyte were normalized to that cell's response to 30 μM ACh alone. Each point represents the average \pm SEM normalized response of at least four cells. The normalized data were then plotted relative to the maximum responses for the various agonists compared with the maximum responses obtained with ACh. Values for the curve fits are given in Table 1. Note that perfusion rates were increased for the nicotine concentration–response measurements (see Materials and Methods). **B:** Residual inhibition or desensitization of subsequent control ACh responses of $\alpha 4\beta 2$ receptors measured 5 min after the agonists had been applied at the indicated concentrations. Data were normalized to each cell's initial response to 30 μM ACh alone. Each point represents the average \pm SEM normalized response of at least four cells. Data not suitable for curve fitting are connected by broken lines for clarity of presentation. The nicotine data were obtained with the faster perfusion rate (Materials and Methods), but no significant differences in residual inhibitions were observed between experiments conducted with normal or fast perfusion (data not shown). **C:** Representative responses of $\alpha 4\beta 2$ -expressing oocytes to the application of 300 μM ACh, RJR-2403, and nicotine. Each trace shows the response to the indicated agonist at a concentration of 300 μM (open bars), flanked by the preceding and subsequent control responses to 30 μM ACh. To indicate the nature of the protracted responses to 300 μM nicotine, the preapplication baselines are indicated as dashed lines. The amplitude of the initial control responses ranged from 1 to 5 μA .

cacy for the $\alpha 3\beta 4$ combination (Fig. 2), suggesting that there is a significant selectivity of RJR-2403 for the $\alpha 4\beta 2$ subtype and that this selectivity is regulated by both the α and the β subunit.

Residual inhibition by RJR-2403 and nicotine

For $\alpha 3\beta 2$ receptors, a residual inhibition was produced by all three agonists when they were applied at high concentrations, with nicotine the most potent at

TABLE 1. Activation curve fits

Receptor	Agonist	Efficacy	Hill slope	EC ₅₀ (μM)
α4β2	ACh	1.2 ± 0.23	0.78 ± 0.23	92 ± 62
α4β2	RJR-2403	1.0 ± 0.07	0.98 ± 0.22	16 ± 4.6
α4β2	Nicotine	0.85 ± 0.06	0.84 ± 0.11	52 ± 13
α3β2	ACh	1.2 ± 0.05	0.81 ± 0.05	370 ± 54
α3β2	RJR-2403	0.38 ± 0.03	0.69 ± 0.06	150 ± 42
α3β2	Nicotine	0.50 ^a	NA	NA
α4β4	ACh	1.0 ± 0.04	1.5 ± 0.12	34 ± 2.2
α4β4	RJR-2403	0.55 ± 0.04	2.4 ± 0.53	50 ± 7
α4β4	Nicotine	0.70 ± 0.04	1.5 ± 0.4	7.8 ± 1.7
α3β4	ACh	1.1 ± 0.12	2.0 ± 0.07	320 ± 6
α3β4	RJR-2403	0.20 ^a	NA	NA
α3β4	Nicotine	0.32 ± 0.03	2.3 ± 1.2	70 ± 20
α3β2α5	ACh	1.0 ± 0.10	0.45 ± 0.10	4.1 ± 2.5
α3β2α5	RJR-2403	0.77 ± 0.25	0.38 ± 0.10	360 ± 700
α3β2α5	Nicotine	0.76 ± 0.16	0.45 ± 0.17	8.8 ± 11.8
α7	ACh	1.0	3.1 ± 0.64 ^b	37 ± 3.0 ^b
α7	RJR-2403	0.16 ± 0.02	3.2 ± 1.8	240 ± 40
α7	Nicotine	0.82 ± 0.05	0.7 ± 0.1 (1.7 ± 0.14) ^c	100 ± 300 (8 ± 25) ^c

NA, not available, data not curve fit.

^a Efficacy approximated from ratio of maximum response to ACh maximum.^b From Papke and Thinschmidt (1998).^c Approximate concentration correction applied (Papke and Thinschmidt, 1998).

producing this effect. Only nicotine produced significant residual inhibition (or desensitization) of α4β4 receptors. Note that, for simplicity, the data for inhibitory effects have been fit to one-site models. However, these agonists may have multiple inhibitory or desensitizing effects, so that the data might be better fit by more complex models (not shown).

No significant residual inhibition of control ACh responses was detected 5 min after the application of any of these agonists to α3β4 receptors. However, inspection of the response waveforms suggests that an alternative form of readily reversible inhibition may limit the apparent efficacy of both RJR-2403 and nicotine with these

as well as α4β4 receptors (Fig. 3). We have previously shown (Papke and Thinschmidt, 1998) that for most β subunit-containing receptors, the time course of the macroscopic ACh response follows the kinetics of solution exchange in the experimental chamber. In Fig. 3, each experimental response is compared with a scaled representation of the respective ACh control responses from the same cells. For the α3β4 and α4β4 receptors (Fig. 3A), at concentrations of <1 mM, the responses to ACh and RJR-2403 follow the kinetics of the ACh control responses closely. The responses to the 1 mM applications, in contrast, reach a rapid peak and then show additional late-phase current. This suggests that peak responses are being limited by some concentration-driven inhibitory process such as desensitization or by secondary noncompetitive inhibitory effects such as channel block. Evident in the response to 1 mM RJR-2403 is an inflection point in the current recording that occurs at a time when drug concentration begins to fall in the chamber. At that time, there appears to be a relaxation out of the inhibited state. Similar waveforms can be seen when the noncompetitive antagonist mecamylamine is co-applied with ACh to some receptor subtypes (Francis and Papke, 1996). Similar effects are observed with nicotine at lower concentrations, with a very pronounced relaxation current following the application of 1 mM nicotine.

It should be noted that these likely noncompetitive effects may account for some reduction in the apparent efficacy of RJR-2403 for α3β4 and α4β4 receptors. However, true partial agonism is also likely to play a role in limiting the maximum responses to this drug. In the case of α3β4 receptors, at all but the highest concentrations, the response waveforms are not very different from

TABLE 2. Residual inhibition

Receptor	Agonist	IC ₅₀ (μM)
α4β2	ACh	NSI
α4β2	RJR-2403	NSI
α4β2	Nicotine	150 ± 75
α3β2	ACh	1,640 ± 640
α3β2	RJR-2403	1,040 ± 290
α3β2	Nicotine	200 ± 600
α4β4	ACh	NSI
α4β4	RJR-2403	NSI
α4β4	Nicotine	79 ± 28
α3β4	ACh	NSI
α3β4	RJR-2403	NSI
α3β4	Nicotine	NSI
α3β2α5	ACh	550 ± 210
α3β2α5	RJR-2403	1,600 ± 670
α3β2α5	Nicotine	18 ± 6
α7	ACh	NSI
α7	RJR-2403	NSI
α7	Nicotine	150 ± 50

NSI, no significant inhibition of subsequent ACh control responses.

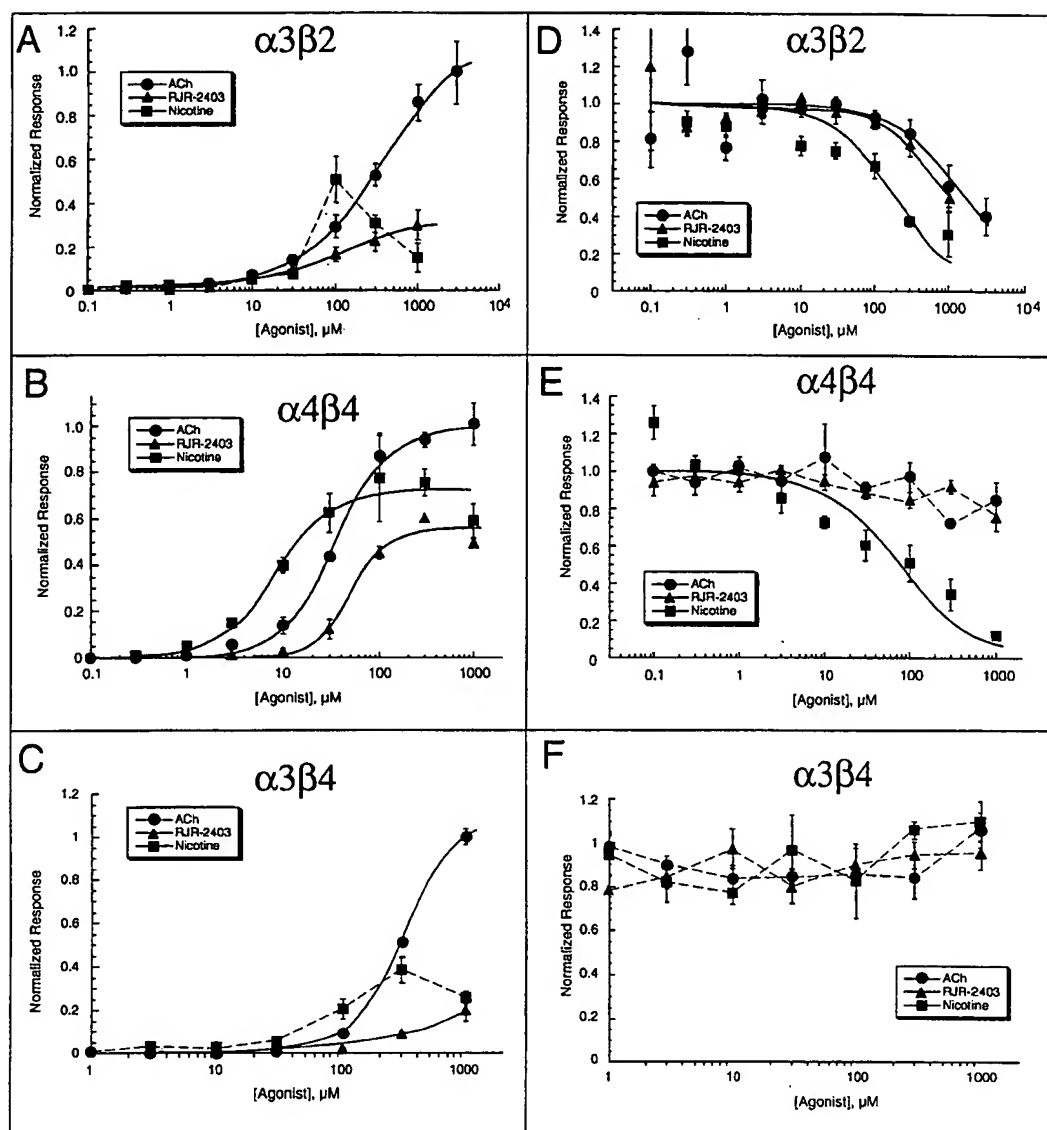


FIG. 2. Concentration-response curves for ACh, RJR-2403, and nicotine on human $\alpha 3\beta 2$ receptors (A), $\alpha 4\beta 4$ receptors (B), and $\alpha 3\beta 4$ receptors (C). Data from each oocyte were normalized to that cell's response to control ACh applications. The ACh controls were 100 μM for $\alpha 3\beta 2$ -expressing oocytes and 30 μM for $\alpha 4\beta 4$ -expressing oocytes and $\alpha 3\beta 4$ -expressing oocytes. Each point represents the average \pm SEM normalized response of at least four cells. The normalized data were then plotted relative to the maximum responses for the various agonists compared with the maximum responses obtained with ACh. Values for the curve fits are given in Table 1. Data not suitable for curve fitting are connected by broken lines for clarity of presentation. Also shown is residual inhibition or desensitization of subsequent control ACh responses of human $\alpha 3\beta 2$ receptors (D), $\alpha 4\beta 4$ receptors (E), and $\alpha 3\beta 4$ receptors (F). Inhibition was measured as a decrease in control ACh responses measured 5 min after the agonists had been applied at the indicated concentrations. Data were normalized to each cell's initial response to control ACh applications. Each point represents the average \pm SEM normalized response of at least four cells.

the ACh controls but had an amplitude that was a factor of 4 less in amplitude than the responses to ACh at the same concentration (as indicated by the differences in the scaling of the ACh control responses for the 300 μM ACh and RJR-2403 responses in Fig. 3A).

As shown in Fig. 3, both $\alpha 3\beta 4$ and $\alpha 4\beta 4$ responses to RJR-2403 and nicotine appear to be limited by similar concentration-dependent forms of inhibition; therefore, it

is likely that the $\beta 4$ subunit may play a crucial role in this process (Webster et al., 1999).

Protracted activation of $\beta 2$ -containing receptors and low-concentration nicotine effects

Just as the $\beta 4$ subunit appears to play a special role in short-term (i.e., reversible) inhibition by RJR-2403 and nicotine (Fig. 3), accelerated peaks followed by pro-

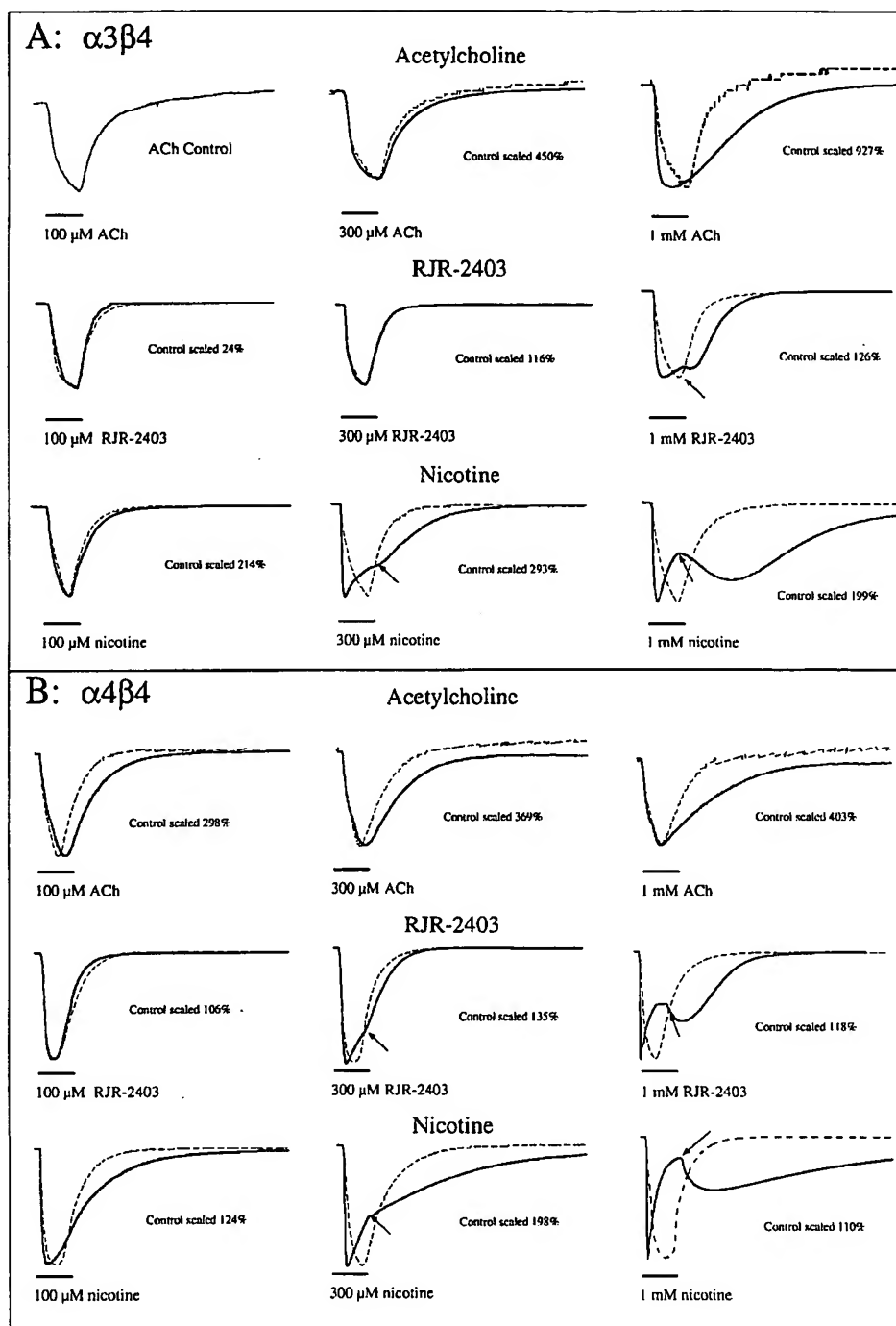
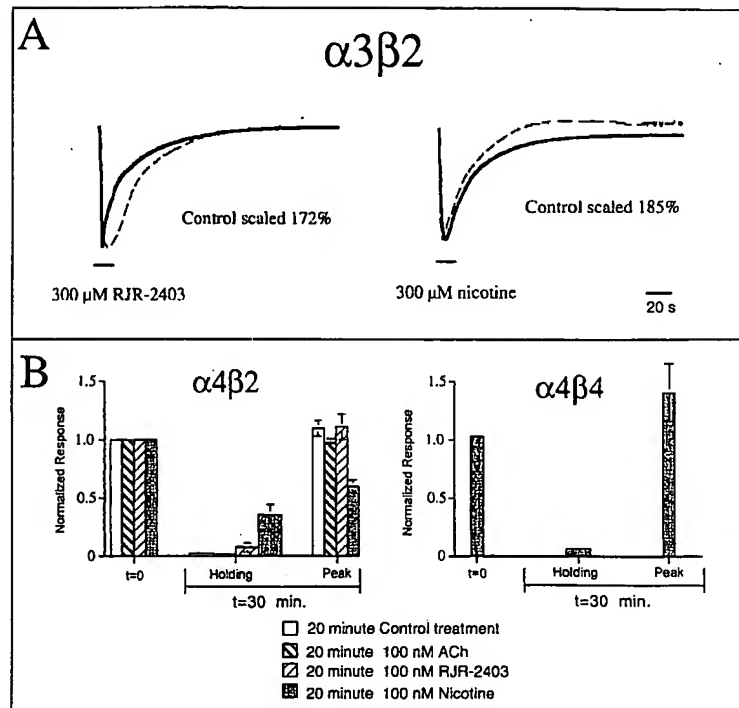


FIG. 3. Representative responses of $\alpha 3 \beta 4$ -expressing oocytes (A) and $\alpha 4 \beta 4$ -expressing oocytes (B) to ACh, RJR-2403, and nicotine at the indicated concentrations (thick lines). Bars below the traces indicate the period of agonist application. Each trace is 200 s of data. Responses are compared with the ACh control responses recorded immediately before the experimental drug applications. Control ACh responses (thin broken lines, 100 μ M ACh for $\alpha 3 \beta 4$ and 30 μ M ACh for $\alpha 4 \beta 4$) have been increased in scale by the indicated factors to facilitate waveform comparisons. The relief of noncompetitive inhibition by agonist is evident (arrows) after the application of either nicotine or RJR-2403 at high concentrations, suggesting that this inhibitory activity may limit the apparent efficacy of these drugs. The amplitude of the control responses ranged from 200 nA to 1 μ A.

longed activation by nicotine are seen with both $\alpha 4 \beta 2$ (Fig. 1) and $\alpha 3 \beta 2$ receptors (Fig. 4A), suggesting that an inhibitory process or unique form of delayed activation

may be limiting the apparent efficacy of nicotine for $\beta 2$ -containing receptors. Exposure to nicotine is also known to induce the high-affinity, presumably desensi-

FIG. 4. A: Representative responses of $\alpha 3\beta 2$ -expressing oocytes to RJR-2403 and nicotine at the indicated concentrations. Responses are compared with the ACh control responses recorded immediately before the experimental drug applications. Control ACh responses have been increased in scale by the indicated factors to facilitate waveform comparisons. Bars below the traces indicate the period of agonist application. Each trace is 200 s of data. **B:** The effects of prolonged exposure to low agonist concentrations in $\alpha 4\beta 2$ - or $\alpha 4\beta 4$ -expressing oocytes. Control 30 μM ACh responses were measured at time zero and used to normalize the holding currents and subsequent ACh responses that followed 20-min applications of the drugs and 10-min wash periods. Note that only nicotine applied to $\alpha 4\beta 2$ receptors produced significant elevations in holding current and decreases in subsequent ACh responses. Increases in $\alpha 4\beta 2$ holding current induced by 100 nM nicotine ranged from 100 to 1,000 nA and were proportionate to the levels of receptor expression (i.e., control ACh responses) in the individual cells. The prolonged incubations with other agonists produced changes in holding current that were usually <100 nA and comparable with the drift in holding current when cells were held in Ringer's solution.



tized state of $\alpha 4\beta 2$ receptors (Fenster et al., 1999; Sabey et al., 1999). It has previously been reported that chronic exposure to low concentrations of nicotine can produce a functional down-regulation of $\alpha 4\beta 2$ receptor function (Olale et al., 1997). We sought to determine whether prolonged exposure to low concentrations of ACh or RJR-2403 would produce either steady-state activation or prolonged inhibition.

Control (30 μM) ACh responses were measured in oocytes expressing $\alpha 4\beta 2$ receptors. The cells were then exposed for 20 min in a static bath to either Ringer's solution or 100 nM ACh, RJR-2403, or nicotine. Following the 20-min incubations, the cells were washed with Ringer's solution for 10 min and then tested for their responses to a second 30 μM ACh application. With this protocol, only the nicotine treatment was observed to produce an inhibition of the second response to ACh (Fig. 4B). The prolonged exposure to nicotine also produced a large increase in holding current required to keep the cells at -50 mV ($n = 5$, $p < 0.01$), measured at the end of the 10-min wash period, whereas neither ACh nor RJR-2403 produced a significant increase in holding current compared with the controls incubated in Ringer's solution. It is tempting to speculate that this slowly developing holding current represents a shift in the concentration dependence for activation by nicotine, arising from an allosteric conversion of receptors to a high-affinity but not yet fully desensitized state. These effects of an exposure to nicotine at low concentration required the $\beta 2$ subunit, as similar effects were not obtained with cells expressing $\alpha 4\beta 4$ subunits (Fig. 4B).

Effect of $\alpha 5$ subunit on $\alpha 3\beta 2$ responses

The nicotinic $\alpha 5$ subunit has been shown to co-assemble with complexes of two or more other subunits and has been reported both to enhance the potency of ACh for activation and to increase desensitization of human $\alpha 3\beta 2$ receptors (Wang et al., 1996). Concentration-response curves for the activation of $\alpha 3\beta 2\alpha 5$ receptors by ACh, RJR-2403, and nicotine are shown in Fig. 5A. Also shown are assessments of residual inhibition produced by these agonists, measured as decreases in the responses to control ACh applications following a 5-min wash. Consistent with previous reports, our data indicate that $\alpha 5$ expression can lead to an apparent increase in the potency of both ACh and nicotine. There is relatively little effect on the apparent potency of RJR-2403 (Table 1). This failure of $\alpha 5$ expression to alter the potency of RJR-2403 may not be taken to indicate that RJR-2403 is effectively stimulating only a subpopulation of $\alpha 5$ -less $\alpha 3\beta 2$ receptors, as $\alpha 5$ expression does produce an increase in the apparent efficacy of RJR-2403 compared with ACh.

We also see that $\alpha 5$ expression increases the potency of ACh and nicotine for secondary inhibition. Significant levels of residual inhibition of $\alpha 3\beta 2$ receptors by nicotine were observed only at concentrations of ≥ 100 μM (Fig. 2), whereas for $\alpha 3\beta 2\alpha 5$ receptors, residual inhibition appears at concentrations of > 10 μM (Fig. 5A). With ACh, we see measurable residual inhibition of $\alpha 3\beta 2$ receptors only at concentrations of ≥ 1 mM, whereas for $\alpha 3\beta 2\alpha 5$ receptors, residual inhibition appears at concentrations of ≥ 100 μM . It is possible that

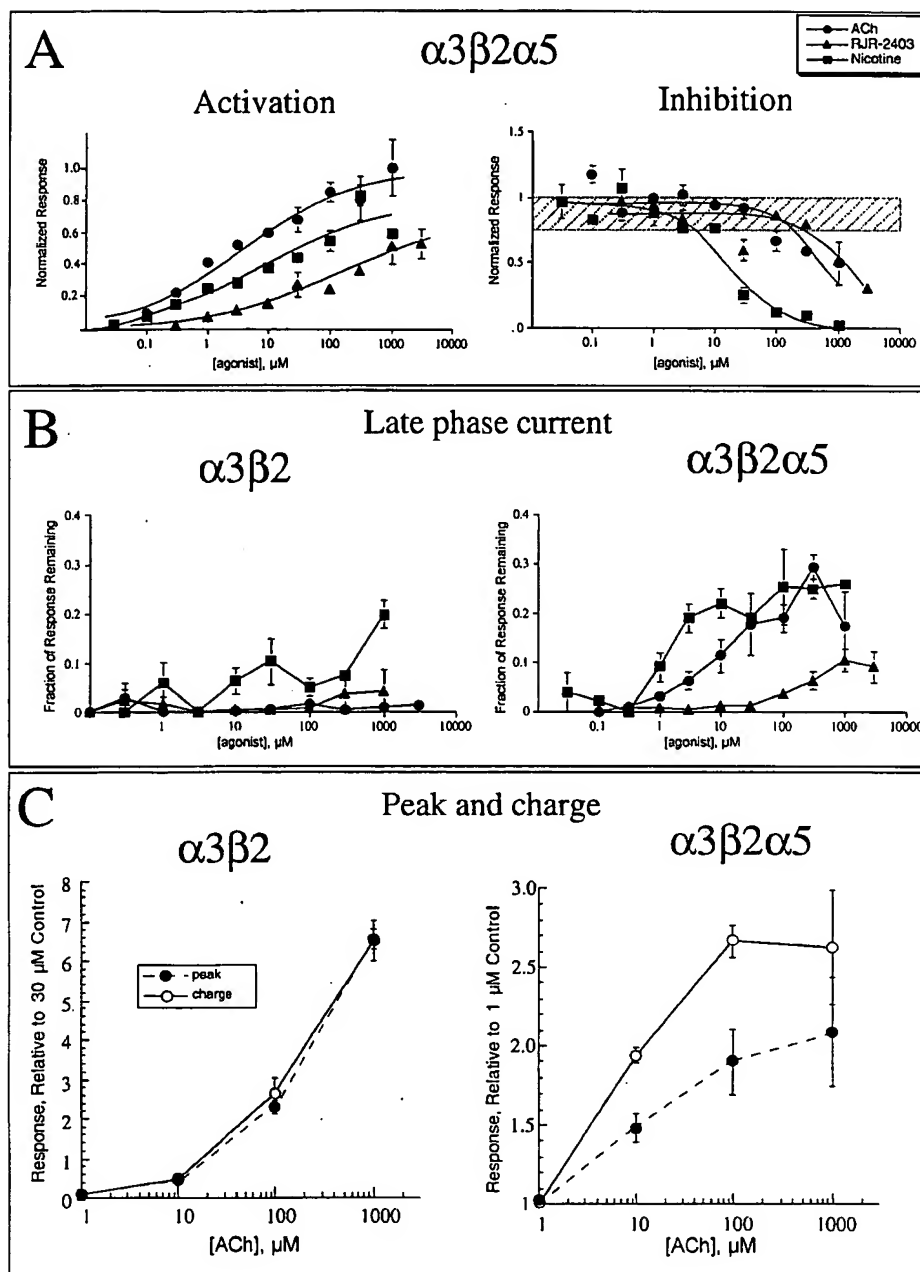


FIG. 5. A: Activation (left) or inhibition (right) of $\alpha 3\beta 2\alpha 5$ -expressing oocytes by ACh, RJR-2403, and nicotine. Data were measured relative to the response to an initial control application of 1 μM ACh 5 min prior to the experimental application. The horizontal hatched bar indicates the criterion value for significant residual inhibition (recovery responses of <75% in our system). B: Residual response of $\alpha 3\beta 2$ - and $\alpha 3\beta 2\alpha 5$ -expressing oocytes measured $t = 2$ min after the start of the response. Values are expressed relative to the peak of the same response. C: Measurements of response as total charge in $\alpha 3\beta 2\alpha 5$ receptors, indicating a small increase in the efficacy of ACh relative to the efficacy calculated from measurements of peak response. Response was calculated as integrated charge over a time course of 3 min immediately following agonist application. Responses were normalized to an initial application of either 30 μM ($\alpha 3\beta 2$) or 1 μM ($\alpha 3\beta 2\alpha 5$) ACh. Peak responses are plotted as a fraction of the maximum total peak response, and charge responses are plotted as a fraction of the maximum total charge response to ACh. Each data point represents the mean \pm SEM response of at least four oocytes.

the apparent increases in the potency of ACh and nicotine may arise, at least in part, from this inhibitory activity, which would limit peak responses at high con-

centration and therefore shift the concentration-response curves to the left. Receptors containing the $\alpha 5$ subunit show an increased late-phase current. Plotted in Fig. 5B

is the residual response at 2 min after the peak, expressed relative to the peak of the same response. We estimated that the residual bath concentration of agonist remaining 2 min after the time of peak response would be $<5\%$ of the original applied concentration, whereas at concentrations of $>1 \mu\text{M}$, the residual current evoked by ACh or nicotine ranged from 10 to 35% of original peak currents.

Neither $\alpha 3\beta 2$ nor $\alpha 3\beta 2\alpha 5$ receptors show any significant residual inhibition 5 min after application of RJR-2403 at concentrations of $<1 \text{ mM}$ (Fig. 5A), and little late-phase current is observed after application of this drug (Fig. 5B). Therefore, it seems likely that the effect of $\alpha 5$ expression on the response of $\alpha 3\beta 2$ -containing receptors to RJR-2403 may be qualitatively unlike the effect of $\alpha 5$ expression on nicotine and ACh responses.

As noted above, an effect of mixed agonist/antagonist activity is depression of peak responses at high agonist concentration. However, an analysis of net charge can indicate that progressively larger responses continue to occur throughout a wider concentration range (de Fiebre et al., 1995; Papke et al., 1997). As shown in Fig. 5C, measures of total charge indicate that the response of $\alpha 3\beta 2\alpha 5$ receptors to ACh continues to increase over a wider concentration range than is seen for concentration-response relationships based on peak amplitude. Thus, one effect of $\alpha 5$ expression, at least in the case of $\alpha 3\beta 2\alpha 5$ receptors, is to make the effects of ACh more similar to those of nicotine in regard to late-phase current and residual inhibition.

Human $\alpha 7$ receptors

The agonist activity of RJR-2403 for human $\alpha 7$ receptors was compared with that of ACh and nicotine. The apparent concentration-response relationships, based on peak responses to various applied agonist concentrations, are shown in Fig. 6. Although such an analysis is useful for purposes of comparison with other published studies (Peng et al., 1994; Meyer et al., 1998a,b), we have previously shown (Papke and Thinschmidt, 1998) that due to the rapid concentration-dependent desensitization of $\alpha 7$ receptors, these plots certainly overestimate EC_{50} values. Also, as previously published, although there is a profound desensitization of the $\alpha 7$ receptors during the application of high ACh concentration, the receptors recover fully from the ACh-induced desensitization (Meyer et al., 1998a). Our data indicate that nicotine has an apparent efficacy for $\alpha 7$ that is roughly equivalent to that of ACh, whereas RJR-2403 is a poor activator of these channels (Fig. 6A). Note that two sets of EC_{50} values are provided in Table 1 for nicotine. One set of values have been corrected for the effects of desensitization, as previously described (Papke and Thinschmidt, 1998). This correction is based on estimates of instantaneous agonist concentration at the time that peak responses are measured, and it is calculated from the comparisons of agonist-evoked responses and solution flow dynamics. Flow dynamics were estimated from open-tip junction potential measurements

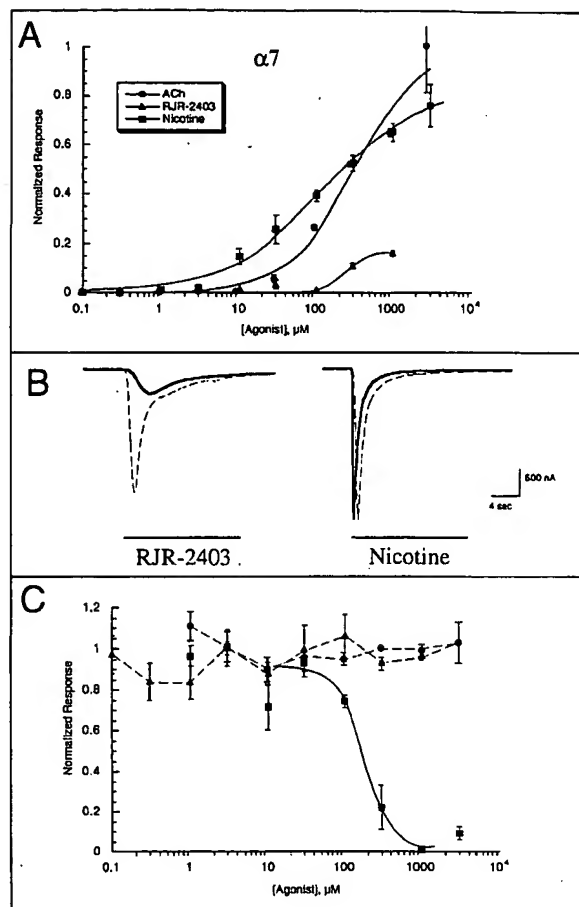


FIG. 6. **A:** Concentration-response curves for the applied concentrations of ACh, RJR-2403, and nicotine on human $\alpha 7$ receptors expressed in *Xenopus* oocytes. Data from each oocyte were normalized to that cell's response to 300 μM ACh alone. Each point represents the average \pm SEM normalized response of at least four cells. The normalized data were then plotted relative to the maximum responses for the various agonists compared with the maximum responses obtained with ACh. The ACh data were taken from Papke and Thinschmidt (1998). Nicotine responses were corrected for instantaneous concentration (Papke and Thinschmidt, 1998), and the corrected values for the curve fits are given in Table 1. **B:** Representative responses of oocytes expressing human $\alpha 7$ receptors to either 300 μM RJR-2403 (left) or 300 μM nicotine (right). The corresponding control responses to 300 μM ACh are shown as the thin dashed traces. The bars underneath the currents represent the duration of the agonist applications. Note that full concentration exchange is achieved only toward the end of the agonist application. **C:** Residual inhibition or desensitization of subsequent control ACh responses of $\alpha 7$ receptors measured 5 min after the agonists had been applied at the indicated concentrations. Data were normalized to each cell's initial response to 30 μM ACh alone. Each point represents the average \pm SEM normalized response of at least four cells. Note that the data are plotted based on the concentrations of agonist applied to the chamber.

made at 20 points around an oocyte placed in the recording chamber.

The kinetics of the $\alpha 7$ responses to RJR-2403 are relatively slow, similar to the responses to ACh at low

concentration, and therefore did not require a correction factor (Fig. 6B) (Papke and Thinschmidt, 1998). In contrast, responses to nicotine deactivate or desensitize even faster than those to ACh (Fig. 6B) and require a 12-fold correction factor in estimated EC_{50} . Although nicotine produced a profound residual inhibition of subsequent ACh control responses, RJR-2403 produced no residual inhibition of ACh control responses (Fig. 6C).

DISCUSSION

The intellectual challenge of ascribing functional roles to the various nicotinic receptor subtypes in the brain is enhanced by the complex pharmacology of nicotinic drugs. Many of the experimental new nicotinic agents being considered for clinical development, including GTS-21, ABT-418, ABT-089, and SIB 1553a (Meyer et al., 1997; Papke et al., 1997; Sullivan et al., 1997; Lloyd et al., 1998) are, in fact, only partial agonists for high-affinity nicotinic receptors and share with nicotine the ability to function as both agonists and antagonists. We have examined the effects of RJR-2403 on a series of human nAChR subunit combinations, comparing the effects of RJR-2403 with those of ACh and nicotine. Our data indicate that RJR-2403 is a potent and effective activator of human $\alpha 4 \beta 2$ receptors with a significant selectivity for this receptor subtype. Moreover, RJR-2403 can be distinguished from nicotine and other new experimental agents by the relatively low level of residual inhibition (or desensitization) that occurs after receptor activation.

Our studies further investigated the qualitative aspects of receptor inhibition by nicotine, as affected by the specific subunit composition of the receptor. Description of whole-cell responses to high agonist concentrations, which show an initial fast decay from an early peak response to a later steady-state response, has led to a difficulty in separating the process of channel block from what is often assumed to represent desensitization. To aid in making the distinction between channel block and desensitization, we can refer to the observation that drugs such as nicotine, anabaseine, and ABT-418 protract the duration of responses past the time when significant concentrations of free agonist would be present in the bath (Papke et al., 1997). Under such conditions, when significant responses are measured in the relative absence of agonist, it seems inappropriate to apply the condition of desensitization, because desensitization is more consistent with the loss of response in the continued presence of agonist. In the present study, we have shown that different forms of persistent activation by nicotine can be seen with $\beta 2$ - and $\beta 4$ -containing receptors. We have also described the effects of expression of the $\alpha 5$ subunit on the kinetics of macroscopic responses to ACh and nicotine, such that $\alpha 5$ expression appears to make ACh-evoked responses more like those evoked by nicotine.

As previously reported for rat nAChR (Fenster et al., 1997), we see that human $\beta 2$ -containing receptors are

more sensitive to functional down-regulation by nicotine than are $\beta 4$ -containing receptors and likewise that $\alpha 4$ -containing receptors are more sensitive than $\alpha 3$ -containing receptors. The especially slow (i.e., protracted) responses of $\beta 2$ -containing receptors to nicotine (see Fig. 1C) raise the issue of whether bath-applied nicotine might be partitioning into the membrane during the drug pulse and then coming back out during the wash to produce the late-phase currents we observe. However, the concept that there may be a nonspecific partitioning of nicotine in and out of the membrane seems unlikely to explain the basis for late-phase current, due to differences in the kinetics and specificity of the late-phase currents for different subunit combinations. By comparing the 300 μM nicotine response in Fig. 1C with the $\alpha 3 \beta 4$ responses to 300 μM nicotine in Fig. 3A, we see that $\beta 4$ -containing receptors have rapidly recovering late-phase currents compared with $\beta 2$ -containing receptors. Although we cannot totally exclude the possibility that these differences may arise from complex differences in concentration dependence for activation by nicotine leaking out of the membrane, no such differences are suggested by the concentration dependence of peak currents (compare low nicotine concentrations in Figs. 1A and 2C). Therefore, the subtype-specific differences in nicotine response appear most consistent with the hypothesis that nicotine is being retained at specific allosteric sites on the receptors, such that dissociation rates from these sites determine the kinetics of late-phase current and prolonged inhibition of $\alpha 4 \beta 2$ receptors by nicotine as previously reported (Olale et al., 1997). It may be noted that residual inhibition is often associated with the presence of protracted currents, suggesting that these may be related phenomena, such that nicotine binding at an allosteric site on $\beta 2$ -containing receptors may first promote activation and then prolonged desensitization. Our data indicate that RJR-2403 may associate with the allosteric sites present on $\beta 4$ -containing receptors but not those on $\beta 2$ -containing receptors. Likewise, the expression of $\alpha 5$ subunits appears to enhance allosteric inhibition (and/or desensitization) by ACh.

To fully understand the specificity of experimental nicotinic agents, for both their desirable CNS effects and largely undesirable peripheral side effects, it will be necessary to obtain complete profiles of specificity for both activation and inhibition of the range of nicotinic receptors in the brain, including those that may contain the $\alpha 5$ subunit. By correlating the pharmacological profiles of specific agents with their profiles of behavioral effects, we may achieve the ultimate goal of knowing how to target agents toward therapeutic effects and away from undesired side effects. At the present time, this remains a daunting challenge. For example, whereas positive cognitive effects have been attributed to nicotine, mice entirely lacking in high-affinity nicotinic receptors not only show no cognitive impairment but even perform better in some cognitive tests than control animals (Picciotto et al., 1995). It may therefore be the case that the cognitive effects of nicotine are mediated by the

activation of nicotinic receptor subtypes unperturbed in the knockout mouse, notably the α -bungarotoxin-sensitive $\alpha 7$ subtype. Agonists selective for this receptor subtype have been shown to have positive cognitive effects comparable with those of nicotine, and these effects are blocked by the nonspecific antagonist mecamylamine (Woodruff-Pak et al., 1994; Meyer et al., 1998a,b). Alternatively, or in addition to $\alpha 7$ -mediated effects, it is possible that the effects of nicotine on cognition arise not from the activation of $\alpha 4\beta 2$ -type receptors but from the long-term inactivation of these receptors. However, both of these interpretations may be called into question by the observation that although RJR-2403 reportedly has cognitive effects in rats that are equal to or better than those of nicotine (Lippiello et al., 1996), RJR-2403 is a poor activator of the $\alpha 7$ receptor subtype and produces very little functional inhibition of $\alpha 4\beta 2$ receptors. This might suggest that in the course of brain development in the knockout mouse, anomalous compensations have been made in the nicotinic systems associated with cognition.

Although some correlations between in vitro pharmacology and in vivo effects are not yet fully understood, other correlations may be readily made. For example, the relatively poor agonist activity of RJR-2403 for the $\alpha 3$ -containing receptors, likely to be found in peripheral nervous tissue, is consistent with the published finding that rats treated with RJR-2403 were relatively free of the side effects of nicotine associated with peripheral receptor activation (Lippiello et al., 1996).

In conclusion, RJR-2403 can be distinguished from nicotine by its relative specificity for $\alpha 4\beta 2$ receptors and its overall low profile of antagonist activities. As we try to approach an understanding of the mechanisms of the antagonist activity of nicotinic drugs and their potential therapeutic actions, this experimental agonist is likely to prove to be a valuable tool for unraveling the complex function of brain nAChRs.

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